

Rapid Effects of IAA on Cell Surface Proteins from Intact Carrot Suspension Culture Cells

Received for publication November 5, 1986 and in revised form January 12, 1987

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ABSTRACT

Suspension cultures of carrot (*Daucus carota* L.) which had an absolute requirement for exogenously supplied auxin were grown in medium containing indoleacetic acid (IAA) as the sole auxin source. Putative cell surface proteins were extracted from the intact cells. Resupply of IAA to cultures partially depleted of auxin resulted in rapidly increased activities of three enzyme activities subsequently extracted. Two of the enzyme activities which increased, peroxidase and pectinesterase, have been implicated in the literature as important to cell wall development, structure, and growth. The other enzyme activity which was increased, IAA oxidase, may be involved in the degradation of IAA *in vivo*. Polypeptides in the extracts were found to increase equally as rapidly as the enzymes in response to IAA as determined with sodium dodecyl sulfate-polyacrylamide electrophoretic gels stained with silver. It is not known whether the changes in enzyme and polypeptide levels in the protein extracts were due to auxin effects on protein synthesis, transport, or extractability.

The effects of auxin on cell enlargement and tissue elongation have been the most extensively studied actions of this plant growth substance. It is clear that cell wall growth and extension are the events which limit and allow this enlargement and elongation. While cell wall acidification appears to be very important in the early stages of rapid auxin-induced growth (13), there is increasing evidence that DNA transcription and mRNA translation may play equally important roles in auxin induced growth (10, 12, 25, 29). It is becoming clear that auxin can effect the population of translatable RNA very rapidly (25, 29), but the identity of the translation products of these mRNAs is generally unknown. Further, it is not clear at this point how early in the events of auxin-induced growth proteins play their crucial role in the specific regions of the cell, such as the wall, where they must act.

Two decades ago, the work of Key *et al.* (18), Nooden and Thimann (24), and others showed that inhibitors of translation strongly inhibit auxin-induced tissue elongation. A number of reports have appeared in the literature which indicate that auxin applications to plant tissues cause increases in the activities of a number of enzymes. Among the enzymes reported to be affected are cellulase (3), glucanases and pectic enzymes (5), dextranases (15), invertase (11), β -glucan synthetase (26), IAA oxidase (20), and peroxidase (1). Most demonstrated auxin effects on enzyme activities occurred after hours or days of treatment, and in many

cases herbicidal concentrations of auxin were used.

When attempting to relate auxin-affected enzyme activities to other auxin effects it gives strength to the argument if it can be demonstrated that the affected enzyme was obtained from a region of the plant cell where it could exert its effect. As the cell wall is a region which must be altered in order for cell enlargement to occur, some studies have been undertaken in which auxin affected cell wall proteins are the intended object of study (23). Rarely if ever has it been demonstrated that the proteins under study were indeed from the cell wall, without contaminating cytoplasmic proteins.

By using gentle extraction methods we have selectively obtained proteins putatively from the cell surface while leaving the plasma membrane and its contents intact. We suggest that these are among the proteins which can be in direct contact with the cell wall. Further, we determined that treatment of cultured cells with physiological concentrations of auxin caused rapid quantitative changes in these proteins, including several enzymes, which were subsequently extracted by our method.

MATERIALS AND METHODS

Plant Material. Liquid suspension cultures of carrot (*Daucus carota* L.) were obtained from Dr. Ann Matthyse of the University of North Carolina. The cultures consisted of individual cells and clumps of typically up to about 12 cells. Cultures were maintained in modified Murashige and Skoog medium (7) further modified in that the pH was adjusted to 5.9 prior to autoclaving and IAA was used instead of NAA. Continued culture growth required regular IAA additions as follows; 2 μ g/ml of medium on d 0 through 3 following transfer, and 3 μ g/ml thereafter. Rotary shakers agitated the cultures at 60 rpm, and transfers occurred at from 5 to 7 d intervals. Growth of cultures was determined by both packed cell volume and cell dry weight.

Cultures were partially depleted of auxin in order to determine the effects of IAA resupply on growth, enzymes, and proteins extracted from the cells. To partially deplete cultures IAA supplementation was discontinued 3 d after culture inoculation, and cells were harvested for extraction 2 d after this. Alternatively, replacement of the medium with fresh medium not containing IAA 4 d after inoculation, followed by harvest 1 d after this, gave results which appeared identical to those reported in this paper.

Protein Extraction. All protein extraction procedures were carried out sterilely. Cells contained in from 35 to 200 ml of suspension culture 5 d after transfer were washed extensively with fresh medium (28), and collected on a filter (Miracloth, Calbiochem-Behring). Collected cells were suspended in a volume of 0.1 M CaCl₂ equal to the volume of medium they were grown in. The suspension was placed on a rotary shaker at 60 rpm for 15 min prior to centrifugation at 400g. The supernatant was dialyzed against cold 50 mM sodium phosphate (pH 6.0)

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(dialysis was against 50 mM NaCl when preparing extract for pectinesterase assay). Dialyzed extracts were concentrated approximately 10-fold within dialysis tubing using carboxymethyl cellulose (Aquacide 1A, Calbiochem-Behring), followed by additional dialysis. Protein content of extracts was determined by the Bradford assay (2) using catalase as standard. The concentrated extracts contained from 250 to 450 μg protein/ml by this method.

Enzyme Assays. All assays were carried out at 25°C. Pectinesterase was assayed by a novel microassay (25) which utilized a pH sensitive dye to achieve a large increase in sensitivity and convenience over the general macroassay method of Kertesz (17) from which the microassay was modified. The assay mixture contained from 3 to 7 μg of extract protein, 1% pectin (Sigma P-9135), 0.4 mg bromocresol purple, and 100 mM NaCl in a total volume of 2.1 ml. The initial pH of this solution was adjusted to 6.8 prior to protein addition, after which the drop in pH occurring upon enzymic deesterification of pectin caused decreased absorbance of the dye at 590 nm. Activity was quantitated by comparison to commercial enzyme of known activity. One unit of activity is defined as the amount of enzyme which will release 1.0 microequivalent of acid from pectin per min at pH 6.8 and 25°C. A similar assay has recently been described elsewhere (14).

Peroxidase activity was assayed by a method using 4-aminoantipyrine as hydrogen donor (6). Extract containing 5 to 15 μg protein was used for each assay.

IAA oxidase assay mixtures contained 17 to 38 μg protein per ml, 1.2×10^{-7} M IAA containing 12,000 cpm of [$1\text{-}^{14}\text{C}$]IAA, 0.43 mM H_2O_2 , and 28 mM K-phosphate (pH 7.0). The rate of reduction of cpm in the solution as determined by scintillation counting was used to calculate the specific activity, expressed as $\text{mol} \times 10^{-7}$ of IAA decarboxylated/h \cdot mg protein.

Alcohol dehydrogenase and malate dehydrogenase were assayed (6) in both CaCl_2 extracts of intact cells (see above) and in CaCl_2 extracts of cells ground in a mortar and pestle. In each case 30 μg of extract protein was used per assay.

Gel Electrophoresis. Concentrated protein samples were denatured in SDS and loaded on gels containing SDS and 10% acrylamide (19). Gels were stained with silver (22). The mol wt markers used were bovine albumin (66,000), egg albumin (45,000), trypsinogen (24,000) and β -lactoglobulin (18,400 subunit).

Determination of IAA Decarboxylation in Cultures. The decarboxylation of IAA in growing cultures was estimated by including a small amount of [$1\text{-}^{14}\text{C}$]IAA in the IAA solution supplied (1.7×10^{-5} M final concentration) to 5 d old cultures. Representative culture samples containing both cells and medium were periodically removed and radioactivity was determined by scintillation counting. The number of counts lost over time was used as an estimate of the loss of the carboxyl carbon from added IAA.

Statistical Analysis and Reproducibility. Where statistical significance is referred to the Student's *t* test (21) was used with 95% confidence level and experiments done in triplicate.

RESULTS

In these studies, we were interested in establishing methods for the extraction of cell surface proteins, without obtaining contaminating proteins from other regions of the cell, which must occur with any procedure which disrupts the plasma membrane. Following the cell surface extraction described, cell condition was evaluated as follows: (a) cells were inspected visually using Nomarski optics microscopy; general condition, absence of plasmolysis, and cytoplasmic streaming rates were evaluated; (b) the percentage of cells excluding Evan's blue dye was determined as an index of culture viability (9); and (c) recovery of cells in fresh

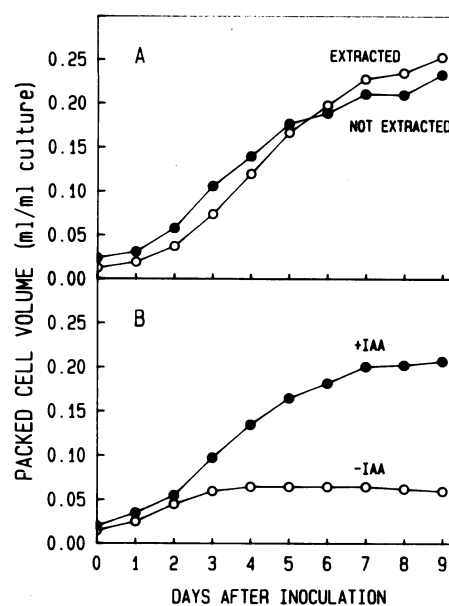


FIG. 1. Growth of carrot suspension cultures following inoculation into fresh medium. A, Packed cell volume increased the same in cultures inoculated with CaCl_2 extracted cells (○) as in those inoculated with cells not extracted (●); B, dependence on exogenous auxin for culture growth; IAA added (●), (see text for details), IAA not added (○). Averages of three experiments are shown for each time point in A and B. In B, time points after 2 h were significantly different. In all cases dry weight determinations (data not shown) were in close agreement with packed cell volume changes.

medium was evaluated through one complete transfer cycle using packed cell volume and cell dry weight as growth indices (Fig. 1A). Cell dry weight closely paralleled packed cell volume (data not shown). None of these three methods of evaluation indicated any adverse affect of the extraction procedure on the condition of the cells. Additionally, the cytoplasmic marker proteins alcohol dehydrogenase and malate dehydrogenase were readily detected in homogenized cell extracts, but were not detected in the cell surface extracts, further demonstrating the efficacy of the extraction method used.

The growth of cultures in the presence and absence of exogenously supplied auxin (IAA) is shown in Figure 1B. Culture growth slowed 2 d after inoculation of cells into auxin free medium, and stopped completely 3 d after inoculation. Both packed cell volume (shown) and cell dry weight (not shown) were equally affected, demonstrating that the cultures used in these studies had an absolute requirement for exogenously supplied auxin. A variety of auxins tested including IAA, NAA,² IBA, *p*-chlorophenoxyacetic acid, 2,4-D, and 2,4,5-T were able to satisfy this auxin requirement. Phenylacetic acid and phenoxyacetic acid were ineffective at any concentration from 0.1 μg to 10 $\mu\text{g}/\text{ml}$.

The rapid time-dependent changes in the activities of pectinesterase and peroxidase following the addition of IAA to partially auxin depleted cultures are shown in Figure 2, A and B. Both pectinesterase and peroxidase activities characteristically increased within 15 to 30 min following auxin addition, and reached peak levels within 1 h. In both cases 2-fold increases in activity were typical. Either a transient or persistent return of activity to about preauxin treatment levels following the initial peak was typical. This pattern was observed in many independent experiments, and in no case failed to develop when the protocol

² Abbreviations: NAA, naphthaleneacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; IBA, indolebutyric acid.

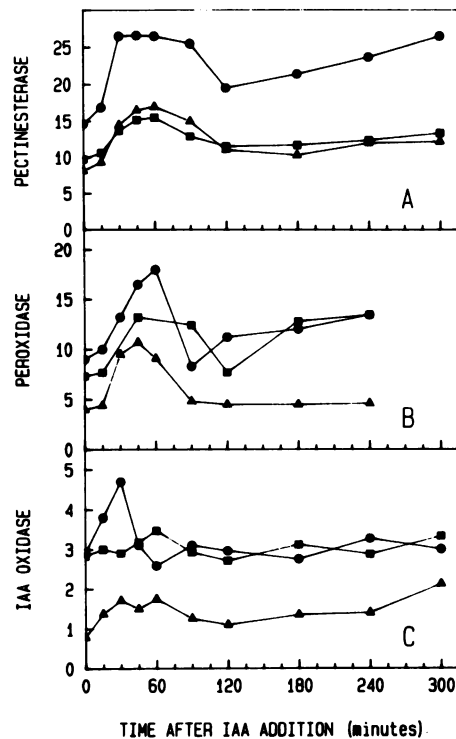


FIG. 2. Rapid increases in enzyme activities following IAA addition to cultures partially depleted of auxin: A, pectinesterase (units: microequivalents acid released/min·mg protein); B, peroxidase (units: $\mu\text{mol H}_2\text{O}_2$ decomposed/min·mg protein); C, IAA oxidase (as indicated by loss of label from $[1\text{-}^{14}\text{C}]\text{IAA}$, units: $\text{mol} \times 10^{-7}$ IAA decarboxylated/h·mg protein). Results of triplicate experiments are shown by ●, ▲, and ■.

described was followed. The activity of these enzymes at periods in excess of 90 to 120 min following auxin addition were quite variable from one experiment to another. Cultures maintained with insufficient auxin for more than 2 d appeared to be in poor condition, failed to exhibit this auxin response, and contained levels of cell surface extractable peroxidase activity many times higher than healthy cultures.

IAA oxidase activity, as indicated by the decarboxylation of labeled IAA, is shown in Figure 2C. While variability was inherently high for this assay, the same general trends in auxin response as observed for pectinesterase and peroxidase were suggested for this activity. As peroxide was required for detection of this activity, a peroxidase enzyme, possibly identical to that producing the activity described above, may have been responsible. The results of the three independent experiments in which this activity was assayed are shown. Although different levels of enzyme activities were found in extracts from different cell cultures, there was no general increase in the amount of protein extracted following auxin treatments.

Proteins extracted from the cell surface by calcium treatment are visible in a silver-stained denaturing electrophoretic gel (Fig. 3). Lanes 1 through 5 are of extracts obtained at times ranging from 0 to 24 h following IAA addition to partially auxin depleted cultures. Two low abundance peptides indicated by arrows, with apparent mol wt of 27,000 and 43,000, consistently increased in intensity following auxin treatment. The lower mol wt polypeptide has been seen to appear within 30 min of auxin treatment in some experiments, while the higher mol wt polypeptide appears at times approaching 24 h after auxin treatment. In several experiments other nonabundant polypeptides appeared within 30 min to 1 h following IAA treatment, but these faint bands were not resolved on all gels prepared.

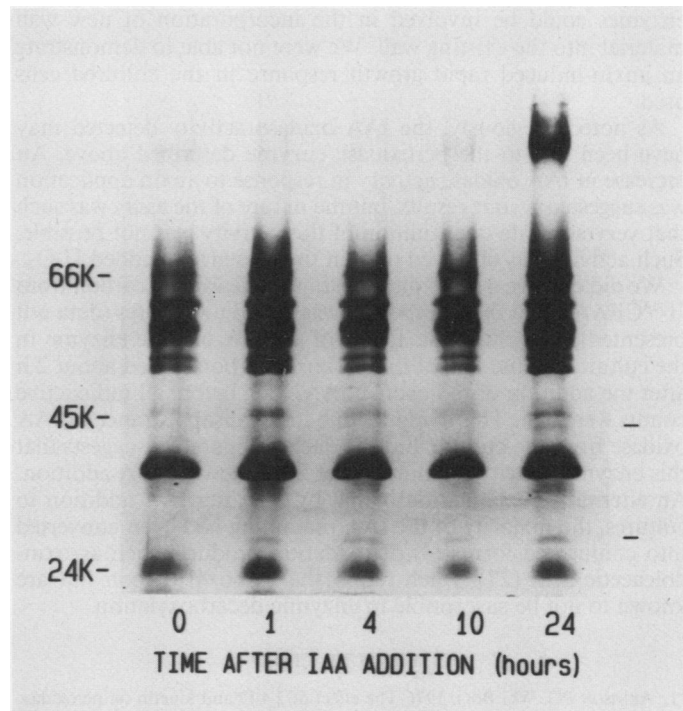


FIG. 3. Changes in electrophoretic pattern obtained from denaturing gels stained with silver; 0, 1, 4, 10, and 24 h following IAA addition to cultures used to prepare protein extracts. Dashes at right indicate bands appearing 1 and 24 h after IAA treatment, with apparent mol wt of 27,000 and 43,000, respectively.

DISCUSSION

The work presented demonstrates that proteins can be extracted from the cell surface of intact cells, without acquiring contaminating proteins from the cytoplasm. The use of suspension culture cells, which made this extraction possible, had further advantages. This material offered a uniform tissue for study, which is not the case when whole plants or tissue sections are used. Further, in suspension cultures, added auxin is able to reach essentially all target cells immediately, as no barriers exist such as those present in intact tissues.

It is known that mRNA transcripts are rapidly altered by auxin in plant shoot tissue (29, 30). However, little information exists as to the nature or site of action of the corresponding proteins presumably produced early in an auxin response. We have shown that increased activities of two enzymes, pectinesterase and peroxidase, become extractable from the cell surface well within 30 min of auxin treatment of carrot cultures. We have not demonstrated increased translation of these enzymes. We have obtained evidence that the activities of these enzymes were increased at the cell surface, a region including the cell wall, which is a likely target for these enzymes.

Pectinesterase converts predominantly methylated pectin to the more highly carboxylated form. The highly carboxylated form of pectin appears to be the most strongly bound in cell walls, presumably through cross-linking via divalent cations ionically bound to the dissociated carboxyl groups (16). In light of this, the increase in cell surface associated pectinesterase activity observed may not be involved in a wall loosening phenomenon.

Peroxidase has been reported to be instrumental in the formation in plant cell walls of isodityrosine, an amino acid which appears to cross-link cell wall glycoproteins (8). As such, cross-linking would increase the rigidity of the cell wall, this enzyme, like pectinesterase, is probably not directly involved in an auxin-mediated cell wall loosening phenomenon. Alternatively, both

enzymes could be involved in the incorporation of new wall material into the existing wall. We were not able to demonstrate an auxin-induced rapid growth response in the cultured cells used.

As noted previously, the IAA oxidase activity detected may have been due to the peroxidase enzyme described above. An increase in IAA oxidase activity in response to auxin application was suggested by our results, but the nature of the assay was such that very accurate quantitation of this activity was not possible. Such activity was observed only in the presence of added H_2O_2 .

We did observe a rapid disappearance of carboxyl carbon from [$1-^{14}C$]IAA when this compound was added to cultures (data not presented), suggesting the action of an IAA oxidase enzyme in the cultures. Rapid loss of this carboxyl carbon ceased about 2 h after the addition of radioactive IAA, long before all radioactive counts were lost. This could be due to the disappearance of IAA oxidase from the culture, but the data in Figure 2C suggests that this enzyme activity remained after 2 h following IAA addition. An alternative explanation is that by 2 h after IAA addition to cultures, the majority of the IAA remaining had been converted into conjugated forms (4) or oxidation products such as oxindoleacetic acid (27) which retain the carboxyl carbon but are known to not be susceptible to enzymic decarboxylation.

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